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MAP Kinase Pathways in Yeast: For Mating and More

Review

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Introduction

MAP kinases (MAPKs) and their upstream regulatory kinases comprise a functional unit that couples upstream input signals to a variety of outputs (reviewed by Blenis, 1993; Blumer and Johnson, 1994; Marshall, 1994). The defining characteristic in this module is the MAPK itself (also called ERK). It has a regulatory kinase, MAPK kinase or MEK (for MAPK/ERK kinase), necessary for its activation. This enzyme is, in turn, regulated by another kinase—raf, mos, or a group of structurally related kinases termed MEKK (for MEK kinase). Raf is found widely in metazoans and is the upstream member of the raf–MEK–MAPK module (Figure 1). Budding yeast lacks raf but has two identified MEK kinases, STE11 and BCK1, which are structurally related to a mammalian MEKK (Lange-Carter et al., 1993). I shall refer to this structurally related group of kinases as the MEKK family. Members of this family have also been identified in tobacco (NPK1; Banno et al., 1993) and in fission yeast (byr2; Wang et al., 1991). These enzymes are upstream components of the MAPK module MEKK–MEK–MAPK (Figure 1). A recent flurry of activity has revealed the manner in which the raf–MEK–MAPK module is regulated by coupling to membrane receptors in a process involving ras and its regulator SOS (reviewed by Blenis, 1993). Little is known in metazoan systems about how MEKK is regulated and about how receptors other than those involving tyrosine kinases feed into MAPK modules.

The budding yeast Saccharomyces cerevisiae has at least six identified pathways that contain MAPKs or their presumed upstream regulators. Studies of these different pathways are providing a wealth of information on different possible inputs to MEKK–MEK–MAPK modules (from serpentine receptors and perhaps from two-component regulatory systems) and on new components that play important roles in the MEKK–MEK–MAPK module and are likely to be found in metazoans (Figure 1). In particular, recent work has revealed the existence of an intriguing novel component, STE5, which appears to be a scaffold for association of the three protein kinases in the MEKK–MEK–MAPK module of the yeast pheromone response pathway. This pathway also contains yet another protein kinase, STE20, which likely functions upstream of the MEKK as a link to the G protein. The existence of mammalian homologs of STE20 ($p65^{PAK}$) raises the possibility that STE20 relatives might also regulate mammalian MEK kinases.

The MEKK–MEK–MAPK modules of budding yeast are involved in a wide variety of biological processes: they govern transitions in its life cycle—mating and invasiveness in haploid strains and pseudohyphal development and spore formation in diploid strains—as well as maintenance of cell wall integrity and response of cells to high osmolarity. A well-studied MAPK module of the fission yeast Schizosaccharomyces pombe is involved in mating and other steps in its life cycle, and an element of a MAPK module has been found in the fungal plant pathogen Ustilago maydis, where it may play a role in responding to signals from its host plant. One of the purposes of this review is to describe the components of these MAPK pathways and to place them in biological context. Studies with these organisms present an opportunity to use facile genetic analysis to identify components of these pathways and to determine whether they play a role in more than one pathway. These studies reveal a case in which a component functions in more than one pathway within a given cell type (Roberts and Fink, 1994) and two examples in which components are functionally redundant (Elion et al., 1991; Irie et al., 1993). These observations and the fact that a unicellular organism has at least six distinct MAPK pathways raise several questions about the specificity with which the MAPKs are activated. Additional goals of this review are to understand the bases for specificity in the different pathways and to highlight some of the new molecular insights into these pathways that may be relevant to similar systems in metazoans. The diversity and function of MAPKs in yeast have been the subject of several excellent recent reviews (Neiman, 1993; Errede and Levin, 1993; Ammerer, 1994).

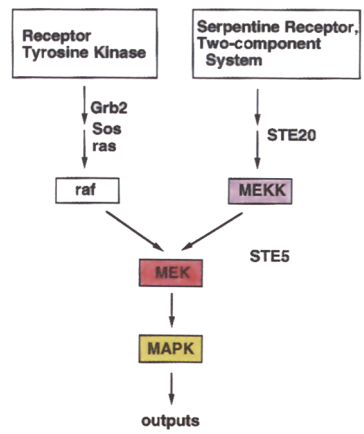

Figure 1. Two Types of MAPK Modules and Their Inputs

The left branch of the diagram describes the raf–MEK–MAPK module as it occurs in mammalian systems, in Drosophila, and in nematodes. In this case, the MEK activity can be stimulated by activating a receptor tyrosine kinase, which leads to activation of the protein kinase raf. Activity of raf is controlled by Grb2, Sos, and ras proteins, which couple the tyrosine kinase to raf. The right branch of the diagram describes the MEKK–MEK–MAPK module as it occurs in yeast, which lacks raf. In this case, activity of the MEKK is controlled ultimately by serpentine (seven membrane-spanning) receptors and possibly by two-component systems, as well as by other mechanisms. The STE20 protein may play a role in transmitting the signal from the serpentine receptor to the MEKK; the STE5 protein appears to be a scaffold for the MEKK–MEK–MAPK module.

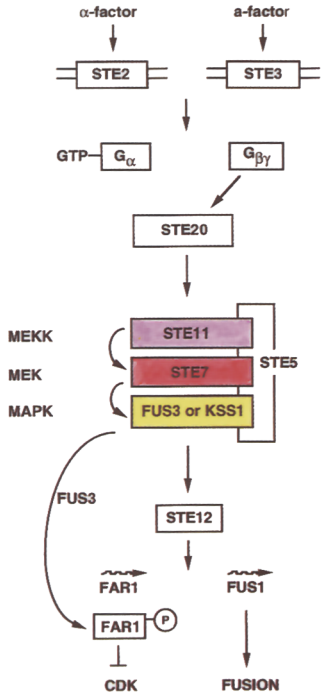

Figure 2. The Pheromone Response Pathway of Budding Yeast

The ligands α factor and **a** factor act on the indicated serpentine receptors in **a** cells (STE2 protein) or in α cells (STE3 protein). Activation of the MAPK cascade leads to phosphorylation of STE12 and FAR1 proteins, which leads to transcriptional induction and cell cycle arrest, respectively.

The Pheromone Response Pathway

The pheromone response pathway is necessary for haploid strains of yeast, which are of mating type **a** or α, to mate with each other. There are three major responses: transcriptional induction of genes involved in mating, arrest of cells in G1, and morphological changes. Each of the haploid cell types produces a peptide mating pheromone, **a** factor or α factor, that acts on the serpentine receptors of its mating partner (Figure 2). **a** cells produce the α factor receptor STE2, and α cells produce the **a** factor receptor STE3; **a**/α cells produce neither. Synthesis of the receptors is governed at the transcriptional level by cell type–specific regulatory proteins; all intracellular components of the pheromone response pathway are common to both **a** and α cells. As described below, the receptors and several of the other components of the pathway are not synthesized in **a**/α cells.

Activation of the receptor causes dissociation of G_α from $G_{\beta\gamma}$, which then activates the MAPK cascade in an as yet unknown manner involving STE20 and STE5 (discussed further below). STE11, the MEKK family member, then phosphorylates and thereby activates STE7, the MEK family member (Neiman and Herskowitz, 1994). STE7, in turn, phosphorylates the MAPKs FUS3 and KSS1 and activates them (Gartner et al., 1992; Errede et al., 1993). The activated MAPKs then apparently activate the transcription factor, STE12, by phosphorylating it (Elion et al., 1993). STE12, often in association with the general transcription factor MCM1, then activates transcription of numerous genes coding for components of the pheromone response pathway itself and genes necessary for cell fusion. Transcriptional activation of these genes by the pheromones has several parallels to gene induction in mammalian systems, in which serum response factor (SRF), whose DNA-binding domain is homologous to MCM1, associates with Elk-1, a MAPK substrate, to induce transcription of genes in response to serum (see Hill and Treisman, 1995 [this issue of *Cell*]).

Cell cycle arrest in response to the mating pheromones results ultimately from action of the FAR1 protein, which binds to cyclin-dependent kinases CDC28-CLN1 and CDC28-CLN2, to inhibit their activity (Peter and Herskowitz, 1994). The activity of FAR1 protein is governed in two ways by the pheromone-response pathway and, in particular, by the MAPK FUS3. First, transcription of the *FAR1* gene is induced 3- to 5-fold by mating pheromones as described above. Second, FAR1 is an excellent substrate for FUS3 in vitro and in vivo (Peter et al., 1993; Elion et al., 1993; Tyers and Futcher, 1993). Because phosphorylation of FAR1 is correlated with cell cycle arrest and inhibition of the cyclin-dependent kinase, it is possible that phosphorylation activates FAR1.

The two MAPKs in this pathway, FUS3 and KSS1, provide an excellent example of functional redundancy: either kinase is sufficient for activation of STE12. This can be seen simply from the observation that mutants lacking either FUS3 or KSS1 (*fus3 KSS1* or *FUS3 kss1* strains) can activate transcription of the *FUS1* gene normally (which requires activated STE12), whereas strains lacking both of these MAPKs (*fus3 kss1* strains) are completely uninducible (Elion et al., 1991). The behavior of the double mutant indicates that FUS3 and KSS1 are the only MAPKs that can activate STE12. It is likewise notable that FUS3 and KSS1 are not completely functionally redundant: FUS3 can phosphorylate FAR1, whereas KSS1 cannot (Peter et al., 1993; Elion et al., 1993).

In addition to FAR1 and STE12, biochemical studies show that FUS3 has several other substrates, including STE7 (Zhou et al., 1993) and STE5 (Kranz et al., 1994). The functional consequences of phosphorylation are not known: it might play a role in down-regulation of the pathway or in activation of the pathway. Mutants of STE7 and STE5 specifically defective in these phosphorylation sites have not been analyzed. The only known substrate for STE11 is STE7, and the only known substrates for STE7 are FUS3 and KSS1.

STE5 Associates with All Members of the STE11-MEK-MAPK Module

Although *STE5* was one of the first four *STE* genes to be identified (MacKay and Manney, 1974), its function has remained enigmatic. The STE5 nucleotide sequence (Leberer et al., 1993; Mukai et al., 1993) reveals an ORF of 917 amino acids but provides no clues to its function. A variety of epistasis tests have been performed with mutations of *STE5* and *STE4*, *STE20*, and *STE11* that indicate that STE5 functions after $G_{\beta\gamma}$ and before STE12 but that leave the relationship to the MAPK cascade ambiguous

(Leberer et al., 1992; Hasson et al., 1994; especially for STE5 and STE11—see Stevenson et al., 1992). Important and unexpected new information on STE5 has recently come from the use of the two-hybrid system (Fields and Song, 1989) and biochemical assays to identify interactions among STE5 and the components of the MAPK cascade (Marcus et al., 1994; Choi et al., 1994; Printen and Sprague, 1994). These analyses are notable not only for their results but also for their comprehensiveness: all pairwise combinations of STE4 (coding for G_β), STE5, STE7, STE11, STE12, STE20, FUS3, and KSS1 were examined by the two-hybrid system. These studies demonstrate that STE5 associates with all of the components of the MAPK module: it associates with STE11, STE7, and with both of the MAPKs, FUS3 and KSS1. In contrast, STE5 does not associate with G_β (STE4), STE20, or STE12. Although failure to find association by the two-hybrid system can occur for many reasons, the interactions observed between STE5 and the members of the MAPK cascade are strong and striking. These in vivo interactions have been supported by two types of biochemical experiments: first, the MAPK components coimmunoprecipitate with STE5 using glutathione S-transferase (GST)-tagged STE5 protein; second, they cosediment at high molecular weights on a glycerol gradient (Choi et al., 1994). Further analysis using the two-hybrid system has given an amazingly simple result: STE11, STE7, and FUS3 or KSS1 appear to associate independently with STE5 and bind to different regions. The independent binding is indicated by the observation, for example, that association of STE5 and STE11 does not require STE7 or FUS3/KSS1 (Marcus et al., 1994; Choi et al., 1994; Printen and Sprague, 1994). The argument that the MAPK components bind to separate domains on STE5 comes from observations on binding to deletion derivatives of STE5. It is observed, for example, that $STE5_{1-336}$ associates with FUS3 or KSS1 but not with STE11 or STE7. In contrast, $STE5_{336-917}$ associates with STE11 and STE7 but not with FUS3 or KSS1 (Choi et al., 1994). These results lead to the proposal that STE5 acts as a scaffold for the MAPK module. In addition to the interactions detected between STE5 and the protein kinases, the two-hybrid analyses indicate that some of the protein kinases associate with each other independently of their association with STE5. In particular, both STE7 and STE11 interact with both MAPKs, FUS3 and KSS1 (Printen and Sprague, 1994; Choi et al., 1994).

The precise functional role of STE5 has not yet been determined. One appealing possibility (discussed further below) is that it activates protein kinases, for example, STE11. The presence of the MAPK components in a complex presents many opportunities for regulation, either for asssembly and activation of the complex or for down-regulation of the cascade. The existence of STE5 as a scaffold for this cascade may provide an answer to the question of how different MAPK modules are kept from interfering in each other's pathways: the association of STE11, STE7 and FUS3 or KSS1 with STE5 may prevent them from associating with other MAPK modules and prevent components of these modules from interacting with STE11, STE7, or FUS3/KSS1 (Marcus et al., 1994; Choi et al., 1994; Printen and Sprague, 1994). In other words, the STE5 scaffold may restrict cross-talk between MAPK cascades.

The biochemical studies described here demonstrate that STE11–MEK–MAPK is not only a functional module but a physical one as well. It would hardly be surprising to find analogs and homologs of STE5 associated with other MEKK–MEK–MAPK modules in yeast and in other organisms. Because STE5 appears to be built of separate domains, it is possible that STE5 analogs are composed of more than one polypeptide chain.

Although it is easiest to imagine a signal transduction pathway to be a simple linear pathway, the existence of a STE5–STE11–STE7–FUS3/KSS1 complex offers a multitude of ways by which components of the pathway can interact both to activate and to dampen function of the pathway. This can create a situation in which epistasis relationships (assessed by the behavior of double mutants with differing phenotypes) can be murky (Stevenson et al., 1992).

Regulation of the MEKK STE11 by $G_{\beta\gamma}$ May Involve Another Protein Kinase, STE20

One of the major unanswered questions about the pheromone response pathway is how $G_{\beta\gamma}$ transmits a signal to activate the MEKK STE11. The argument that $G_{\beta\gamma}$ rather than G_α is responsible for stimulating the pathway is simple: mutants lacking G_β or G_γ (defective in the *STE4* or *STE18* genes, respectively) are uninducible. In contrast, mutants lacking G_α (defective in the *GPA1/SCG1* gene) are constitutive for the pathway (Dietzel and Kurjan, 1987).

The STE20 protein is poised to play an interesting and important role in the pheromone response pathway: it is necessary for signal transmission in the pheromone response pathway (at least in some genetic backgrounds; Leberer et al., 1992; Ramer and Davis, 1993) and appears to function after $G_{\beta\gamma}$ and prior to STE11. Two types of epistasis analysis that lead to this conclusion are the following (Leberer et al., 1992). First, inactivation of *STE20* blocks activation of the pheromone response pathway that results from overexpression of G_β. Second, inactivation of *STE20* has no effect on a constitutively active mutant form of STE11. It has been proposed that STE20 activity may be controlled directly by $G_{\beta\gamma}$ (Leberer et al., 1992), but no data exist on this point. Another possible target for $G_{\beta\gamma}$ is STE5.

STE20 has the structural features of a protein kinase and, indeed, has striking identity to the kinase $p65^{PAK}$: 70% of the residues in a segment of 250 amino acids containing the catalytic domain are identical (Manser et al., 1994). STE11 would be a possible and appealing substrate for STE20.

As for several other protein kinases, including raf and STE20, the amino-terminal segment of STE11 appears to be a negative regulatory domain (Cairns et al., 1992; Stevenson et al., 1992). Deletion of the first half of STE11 (whose total size is 717 amino acids) leads to a constitutively active form of STE11 (Cairns et al., 1992). Amino acid substitutions at positions 279 and 596 (the latter in the kinase domain) also lead to constitutive activity (Stevenson et al., 1992). The amino-terminal segment of

STE11 might inhibit STE11 activity by direct action or by binding to an inhibitor. In either case, phosphorylation of this amino-terminal segment by STE20 could relieve inhibition. It is notable that the amino-terminal segment of STE11 is sufficient for association with STE5, which raises the possibility that STE5 may play a role in activating STE11 (Choi et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994).

The striking similarity between STE20 and its mammalian homolog, $p65^{PAK}$, raises some further intriguing possibilities based on recent observations on regulation of $p65^{PAK}$. Manser et al. (1994) have shown that its activity is stimulated by GTP-bound forms of mammalian Cdc42 and Rac proteins. ($p65^{PAK}$ is thus analogous to raf in that it is a protein kinase whose activity is controlled by a GTPase.) These observations on control of a STE20 relative by GTPases may be relevant to both yeast and mammalian signal transduction systems. The yeast CDC42 protein is necessary for cell polarity (Johnson and Pringle, 1990) and is reported to bind to STE20 (Manser et al., 1994). Control of STE20 activity by CDC42 could link morphogenesis and the pheromone response pathway. (Because yeast contains other genes with similarity to STE20 [Manser et al., 1994], CDC42 might function through a protein other than STE20.) Nothing is known about how mammalian MEKK is regulated (Blumer and Johnson, 1994). Manser et al. (1994) have suggested that $p65^{PAK}$ might control its activity and thereby regulate a mammalian MEKK–MEK–MAPK module. Exciting things are on the horizon for STE20.

The Invasive Response of Haploids and Pseudohyphal Development of Diploids Utilize Several Components of the Pheromone Response Pathway

Yeast not only exists in different cell types, but these cell types exhibit further cell specialization depending on their nutritional status. In particular, it has recently been discovered that haploid strains (of **a** or α cell type) exhibit a distinctive response to nutritional starvation termed the invasive growth response (Roberts and Fink, 1994): cells growing on agar invade the agar and grow beneath the cell surface, so that the colonies remain anchored to the surface. They also exhibit changes in their budding pattern. Amazingly, the ability to exhibit invasiveness requires many of the components of the pheromone response pathway. The requirement of some but not all of the components of the pheromone response pathway is revealing, since it demonstrates that a single cell type can modify its MEKK–MEK–MAPK module.

Invasiveness requires the first two components of the pheromone response MAPK module, STE11 and STE7, and a likely regulator, STE20. It also requires one of the same output components of the pheromone response MAPK cascade, the transcription factor STE12. The lack of requirement for the receptors and for the heterotrimeric G protein presumably reflects a different input for this pathway. As noted above, it is plausible that STE20 is an important conduit of information from the activated receptor via $G_{\beta\gamma}$ for the pheromone response pathway. The invasive pathway presumably has some type of signal, perhaps intracellular but conceivably extracellular, that feeds into the invasive response pathway via STE20.

The behavior of mutants defective in *KSS1* or *FUS3* is not simple and points to the existence of other MAPKs specific for this pathway. In particular, *kss1 FUS3* mutants are defective for invasiveness, and *KSS1 fus3* mutants exhibit enhanced invasiveness. Surprisingly, mutants defective in both genes exhibit normal invasiveness. These observations indicate that KSS1 and FUS3 are not required for the invasive behavior but modulate it. Since the invasive growth response requires neither of the MAPKs of the pheromone response pathway, nor does it require several other MAPKs (such as those involved in osmotic response and in cell wall integrity), it is conceivable that this pathway uses no MAPK. A more attractive possibility is that this pathway employs a distinctive MAPK (Figure 3). It would be further appealing if this pathway also involved a pathway-specific STE5 analog (Figure 3) (Roberts and Fink, 1994).

The existence in haploid cells of two distinctive pathways that use many of the same components (STE20, *STE11* MEKK, *STE7* MEK, and STE12) raises many questions, including how the output responses governed by the transcription regulator STE12 become different in the two situations. Haploid cells of yeast thus offer the same mechanistic challenges as mammalian PC12 cells, which exhibit different responses to different growth factors (discussed further by Roberts and Fink, 1994; Marshall, 1995 [this issue of *Cell*]).

a and α cells exhibit a much stronger invasive growth response than **a**/α cells (Roberts and Fink, 1994). This observation can be explained if **a**1–α2, the repressor characteristic of **a**/α cells, turns off a pathway-specific component such as the MAPK or STE5 analog.

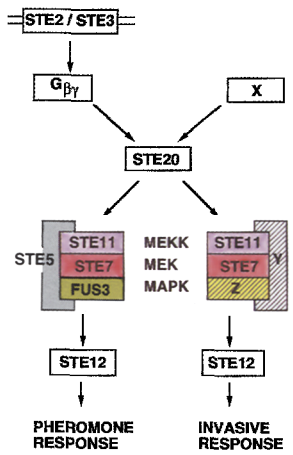

Figure 3. Common Components Are Involved in the Pheromone Response Pathway and in the Invasive Growth Response Pathway

The invasive growth response requires STE20, STE11, STE7, and STE12, but not STE5. The MAPK module involved in the pheromone response pathway is associated with STE5. The MAPK module involved in the invasive growth response may use an analog of STE5 (Y) and and analog of FUS3 (Z). **a**/α cells require STE20, STE11, STE7, and STE12 for pseudohyphal development and may also employ distinctive STE5 and MAPK analogs.

In contrast with their modest invasive response, **a**/α diploid cells exhibit a unique response not exhibited by haploids: pseudohyphal differentiation (Gimeno et al., 1992). This is a multifaceted program in which cells change shape from ovoid to sausage-like, exhibit unipolar budding and remain connected to form chains resembling the hyphae of filamentous fungi, and exhibit an altered cell cycle (Gimeno et al., 1992; Kron et al., 1994).

The ability of cells to exhibit pseudohyphal development has the same spectrum of requirements as the invasive response, with the exception of a more straightforward behavior of mutants lacking the MAPKs: mutants defective in *FUS3*, *KSS1*, or both exhibit normal pseudohyphal behavior (Liu et al., 1993). Comparison of the pheromone response pathway and the pseudohyphal development pathway offers some interesting contrasts. Because these processes occur in different cell types, it is important to note first what components are differentially synthesized. **a**/α cells turn off synthesis of several components of the pheromone response pathway, notably the receptors, the three subunits of the G protein, STE5, and FUS3. In addition, synthesis of STE12 is turned down around 5-fold, at least in some strain backgrounds (Fields and Herskowitz, 1987; Liu et al., 1993). Since *STE5*, *FUS3*, and several other genes are turned off in **a**/α cells, it is to be expected that they are not required for pseudohyphal development. In contrast, STE7 and STE11 are expressed in all cell types, and their role in **a**/α cells has been a mystery. We now see that they, along with STE20 and STE12, are necessary for pseudohyphal development. The independence of the pseudohyphal development pathway from the pheromone response pathway components, STE5 and its associated MAPKs, can be nicely accounted for if **a**/α cells contain pseudohyphal pathway–specific analogs (Gimeno et al., 1992).

Pseudohyphal development is triggered by starvation, and presumably at least some input signals feed into the pathway via STE20 (Figure 3). The yeast RAS proteins may provide some of this input, as indicated by the fact that mutants with activated forms of RAS exhibit psdeudohyphal development on rich medium (Gimeno et al., 1992). Numerous morphological changes occur during pseudohyphal development. It is conceivable that there is a functional link between STE20 and CDC42, which is involved in morphogenesis, that might play a role in mediating some of these morphological alterations. It is striking that the outputs of both the invasive growth and the pseudohyphal development pathways involve STE12, which occupies a similar position in the pheromone response pathway. The basis for the difference in action of STE12 in these different situations remains to be determined.

A MAPK Cascade Controls Response to High Osmolarity and May Be Regulated by a Two-Component System

When yeast cells are confronted with high osmolarity, they induce synthesis of glycerol to increase their internal osmolarity. The *PBS2* and *HOG1* genes, which code for a MEK homolog and a MAPK homolog, respectively, are necessary for cells to grow at high osmolarity (Brewster et al., 1993). Tyrosine phosphorylation of the *HOG1* MAPK is rapidly induced when cells are exposed to increased external osmolarity (Brewster et al., 1993). HOG1 may activate a transcription factor analogous to STE12 that governs transcription from the high osmolarity regulatory element (Schüller et al., 1994; Albertyn et al., 1994).

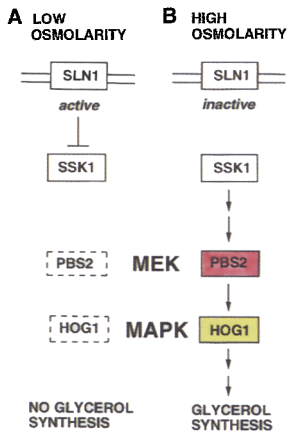

Figure 4. Control of Glycerol Synthesis by the HOG Pathway

(A) Under conditions of low osmolarity, SLN1, the putative kinase component of the two-component system, is active and inhibits function of SSK1. The *PBS2* MEK and the *HOG1* MAPK are thereby inactive. (B) Under conditions of high osmolarity, SLN1 is inactive, which allows SSK1, a putative receiver component of this two-component system, to be active. SSK1 leads to activation of the *PBS2* MEK and *HOG1* MAPK and to glycerol synthesis. This pathway is termed the HOG pathway because it controls high osmolarity glycerol response.

Although a MEKK for this pathway has not yet been reported, candidates for components that function early in the pathway have been identified. These products may comprise a eukaryotic version of a two-component regulatory system like those widespread in bacteria. The yeast *SLN1* gene was identified in a hunt for mutants that cannot tolerate a defect in proteolysis (Ota and Varshavsky, 1993). This gene contains an open reading frame with striking similarity to bacterial two-component regulators. In this case, SLN1 contains two putative transmembrane domains, suggesting that it is an integral membrane protein receptor. It also contains regions that are similar to the sensor histidine kinase and to the response regulator domains of bacterial two-component systems. Several bacterial proteins contain both domains in a single polypeptide (Ota and Varshavsky, 1993). The function of SLN1 and the signal to which it responds were unknown until the recent work of Maeda et al. (1994) describing the growth defect exhibited by mutants lacking the *SLN1* gene. They observed that mutations in any of four genes, including *HOG1*, *PBS2*, and a novel gene termed *SSK1* (for suppressor of sensor kinase), allowed growth in the absence of *SLN1*. Since *HOG1* and *PBS2* are components of a signal transduction pathway required for response to high osmolarity, it is proposed that SLN1 controls activity of the *PBS2* MEK and the *HOG1* MAPK (Maeda et al., 1994) (Figure 4).

The nucleotide sequence of the *SSK1* gene reveals that it too has striking similarity to bacterial response-regulatory domains (Maeda et al., 1994). The SSK1 protein ap-

pears to function prior to HOG1 (and presumably prior to the *PBS2* MEK), because overproduction of SSK1 induces tyrosine phosphorylation of HOG1. For this reason and because of the similarity of SSK1 to two-component regulators, it has been proposed that the putative receptor/protein kinase component, SLN1, controls activity of SSK1. Because inactivation of SSK1, PBS2, or HOG1 reverses the lethality exhibited by mutants lacking SLN1, it is proposed that SLN1 inhibits SSK1 and that high osmolarity inhibits SLN1, thereby allowing SSK1 to stimulate the *PBS2* MEK and the *HOG1* MAPK.

The inviability of strains deleted for SLN1 can be reversed not only by inactivating the protein kinases PBS2 and HOG1 but also by overproduction of several different protein tyrosine phosphatases (PTP2, PTC1, or PTC3) (Ota and Varshavsky, 1993; Maeda et al., 1994). It has therefore been proposed that these phosphatases might inactivate PBS2 or HOG1.

Cell Wall Integrity Requires a PKC and a Complete MEKK-MEK-MAPK Module

Yeast contains one protein kinase C (PKC) that has been studied biochemically and genetically (Levin et al., 1990; Paravicini et al., 1992; Watanabe et al., 1994; Antonsson et al., 1994). This PKC, encoded by the *PKC1* gene, is a homolog of the α, β, and γ isoforms of mammlian PKC. Unlike the mammalian enzymes, this yeast PKC activity does not exhibit any dependence on phospholipid, although it is possible that suitable in vitro conditions have not yet been found (Watanabe et al., 1994). An apparently distinct yeast PKC activity has been reported that is stimulated by phospholipid (Ogita et al., 1990), but mutants defective in this gene have not been described. A second PKC-encoding gene has recently been reported, but doubt has been cast on its existence (Levin et al., 1994a).

Mutants lacking *PKC1* exhibit at all temperatures a cell lysis defect that can be suppressed by medium of high osmolarity (for example, 1 M sorbitol) (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). Growth of PKC1-deficient cells under these conditions indicates that a major role of PKC1 is to ensure integrity of the cell wall.

The components of the pathway in which PKC1 functions have been identified in diverse mutant hunts, including those for cell lysis (Torres et al., 1991; Martin et al., 1993) and for enhancement of the growth defect of *cdc28* and other mutants (Mazzoni et al., 1993; Costigan et al., 1992). PKC appears to be a specific target of the antifungal agent staurosporine, and it has been observed that one class of staurosporine-sensitive mutants is defective in *PKC1*. Another class of staurosporine-sensitive mutants whose growth defect is suppressed by high osmolarity defines the *STT4* gene, which codes for a putative phosphatidylinositol 4-kinase (Yoshida et al., 1994). These observations raise the interesting possibility that phosphoinositide metabolism governs the yeast PKC1 pathway.

Several of the components of the PKC1 pathway have been identified by isolation of suppressors, in particular, high copy plasmid suppressors, that restore growth to mutants defective in different components of this pathway (Lee et al., 1993; Irie et al., 1993; see also Torres et al., 1991; Lee and Levin, 1992) (Figure 5). These studies led to identification of genes for a MEKK homolog, *BCK1*, a MEK homolog, *MKK1*, and a MAPK homolog, *MPK1* (also known as *SLT2*; Torres et al., 1991). The *MKK1* gene turns out to be functionally redundant with a related gene, *MKK2* (Irie et al., 1993). Mutants lacking any of these components—the MEKK (*bck1*), the two MEKs (*mkk1 mkk2*), or the MAPK (*mpk1*)—exhibit the same phenotype: lysis at high temperature. Because the phenotype of mutants deleted for PKC1 is more severe than for mutants defective in the proposed downstream components (*pkc1* mutants having a lysis defect at all temperatures), it has been proposed that PKC1 controls a bifurcated pathway, one arm of which contains the MAPK module (Lee and Levin, 1992).

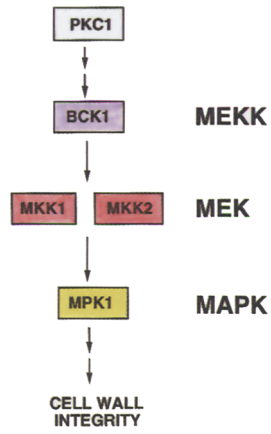

Figure 5. The Components of the PKC Pathway

Activity of PKC1 may be controlled by phosphatidylinositide metabolism, for example, by the PI4 kinase, STT4. The pathway distal to PKC1 is thought to branch; only the branch containing the MEKK–MEK–MAPK module is shown. The components of this pathway have a variety of names: BCK1 is also known as SLK1 and SSP31; MPK1 is also known as SLT2.

It has been reported (Levin et al., 1994b) that PKC1 can phosphorylate BCK1 in vitro. The functional consequences of such phosphorylation are unknown. Targets of the *MPK1* MAPK have not yet been identified. The finding that overproduction of the HMG1-like proteins NHP6A and NHP6B can suppress growth defects of mutants defective in the MEKK or MAPK components of this pathway suggests that these proteins might be substrates for the MAPK (Costigan et al., 1994).

Spore Formation Requires Homologs to STE20 and to MAPKs

Yeast sporulation is the process of meiosis followed by packaging of the haploid nuclei into spores. Sporulation is exhibited only by **a**/α cells and requires nutritional starvation (reviewed by Mitchell, 1994). There are numerous landmark events in meiosis—DNA replication, chromosome synapsis, recombination, the first meiotic segregation, the second meiotic segregation, and finally spore formation. In many cases, these events are paralleled by changes in gene expression: one set of genes (including *HOP1*, which is involved in chromosome pairing) is tran-

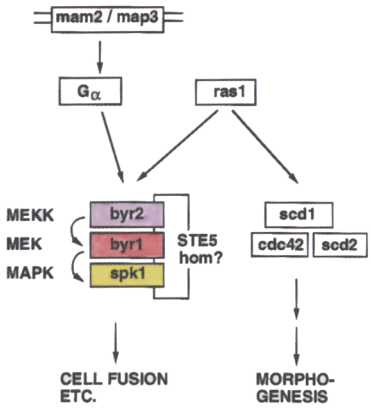

Figure 6. The Pheromone Response Pathway and Dual Roles for ras Protein in Fission Yeast

The pheromone response pathway begins with serpentine receptors (mam2, whose ligand is P factor, or map3, whose ligand is M factor). Ligand stimulates formation of GTP-bound G_α, which activates the MAPK module by an unknown mechanism, perhaps involving input also from ras1 protein. Ras1 protein interacts with a group of proteins (scd1, cdc42sp, and scd2) that are involved in morphogenesis. It is not known whether fission yeast has a homolog to STE5 protein of budding yeast. Figure modeled after Chang et al. (1994).

scribed early in sporulation, another set of genes (including *SPO12*, which is involved in chromosome distribution) is transcribed after the first set, and a third set of genes (including *SPS100*) is transcribed after completion of the second meiotic division. One of the great challenges in understanding this developmental pathway is to determine how the different events are coordinated with each other.

Two yeast genes necessary for spore formation have recently been identified that code for putative components of a MEKK–MEK–MAPK module. In particular, the *SPS1* gene codes for a putative protein kinase that is clearly related to STE20 (44% identical in the catalytic domain) (Friesen et al., 1994). Likewise, the *SMK1* gene codes for a putative MAPK that is 40% identical to FUS3 (Krisak et al., 1994). Several observations indicate that SPS1 and SMK1 participate in a common pathway. First of all, the phenotypes of mutants lacking *SPS1* or *SMK1* are identical in many respects: they both proceed normally through the second meiotic division but then are defective in completing sporulation. The mutants produce defective spores with altered spore walls and express the late group of sporulation-specific genes (such as *SPS100*) at greatly reduced levels. A second striking feature of SPS1 and SMK1 is that their transcription is regulated in a sporulation-specific manner: both are expressed with the middle group of genes such as *SPO12*. These observations lead to the hypothesis that SPS1 and SMK1 participate in a signal transduction pathway necessary for late stages of sporulation, in particular, for spore formation. By analogy with the pheromone response pathway, SPS1 might govern activity of a MEKK–MEK–MAPK module containing SMK1 as its MAPK. Because the two known components of this putative pathway, SPS1 and SMK1, are under transcriptional control, it is possible that the activity of this pathway is determined by the synthesis of these components rather than by controlling their activity (Krisak et al., 1994; Friesen et al., 1994). Further studies of this pathway seem likely to shed light on novel mechanisms for regulating MAPK modules.

The Pheromone Response Pathway in Fission Yeast Has Many of the Same Components as in Budding Yeast and Is Also Regulated by Ras

Like budding yeast, the fission yeast S. pombe also has two mating types, P (h^+) and M (h^-), and corresponding pheromone systems involving receptors with seven presumed membrane-spanning segments, heterotrimeric G proteins, and MAPK cascade components (Figure 6). Because fission yeast is so highly diverged from budding yeast, both the similarities and differences in their pheromone response pathways and modes of sexual differentiation are notable. The similarities in the MAPK cascade are striking and in fact were one of the early observations leading to the appreciation that MEKK, MEK, and MAPK form a functional unit (Neiman et al., 1993). The byr2 protein is homologous to STE11, byr1 to STE7, and spk1 to FUS3 and KSS1. The fission yeast MEK byr1 can partially complement a budding yeast strain deficient in STE7 if the MEKK homolog byr2 is coexpressed (Neiman et al., 1993). These observations indicate that byr2 and byr1 are not only structurally related to STE11 and STE7, respectively, but are also functionally related and that byr2 and byr1 comprise a functional unit. A STE5 homolog has not been reported.

There are a few notable differences between the fission yeast and budding yeast pathways. First, in fission yeast, the G_α subunit of the G protein is essential for triggering subsequent events in the pathway (Obara et al., 1991). Its target has not yet been identified. Second, the overall process of sexual differentiation in fission yeast has an important nutritional input. In budding yeast, the haploid cells express **a**- and α-specific genes for the pheromone response system under all nutritional conditions. In contrast, differentiation of fission yeast cells into specialized cells requires nutritional starvation. The fission yeast ras1 protein is thought to play important roles in this process by stimulating two pathways. It has been observed that ras1 and G_α provide independent inputs into the MAPK module to stimulate some steps in mating (Xu et al., 1994). Ras1 protein interacts with the MEKK homolog byr2 in the two-hybrid assay (Van Aelst et al., 1993; Chang et al., 1994; Van Aelst et al., 1995). Such an interaction is analogous to that observed between ras and raf (see, for example, Van Aelst et al., 1993). Whether ras1 stimulates activity of byr2 is not known. Ras1 may also stimulate a pathway involved in cell morphogenesis (Chang et al., 1994) (Figure 6): in vivo analysis using the two-hybrid system and in vitro biochemical assays indicate that ras1 can interact with the scd1 protein, which is homologous to a budding yeast protein known to be required for cell morphogenesis. The scd1 protein, in turn, interacts with other proteins (cdc42sp and scd2), which are also homologs of proteins known to be involved in cell morphogenesis.

A Presumptive MEK of U. maydis Is Involved in Pheromone Response and Tumor Induction

The signaling systems described thus far in budding and fission yeasts are involved in life cycle transitions and in environmental monitoring. Cell–cell interactions of course also play many important roles in the development of multicellular organisms. They are also expected to play a role in another fundamental biological process: interactions between infectious agents and their hosts. Genetic analysis of the fungal plant pathogen U. maydis may have identified a component of a MAPK cascade involved in such signaling.

The life cycle of U. maydis is considerably more elaborate than those of unicellular fungi such as budding and fission yeasts. It can exist as a haploid, yeast-like form or as a dikaryotic, filamentous form that is pathogenic (reviewed by Banuett, 1992). Cell–cell interactions of haploids are governed by the *a* locus, which has two alleles (Trueheart and Herskowitz, 1992; Snetselaar, 1993; Spellig et al., 1994; Banuett and Herskowitz, 1994b). Each allele codes for pheromone precursor genes and receptors related to budding yeast **a** factor and its receptor STE3 (Bölker et al., 1992). The pheromone system also appears to be necessary for properties of the filamentous dikaryon: maintenance of the filamentous form requires that both *a* alleles be present (Banuett and Herskowitz, 1989; Bölker et al., 1992). Thus, the phermones of the *a* locus appear to control not only a paracrine response between *a1* and *a2* haploid cells but an autocrine response of the filamentous form.

Given the existence of pheromones and receptors such as those of yeasts, it was anticipated that Ustilago might have components of a MAPK module. Recently, a Ustilago MEK homolog, fuz7, has been identified (Banuett and Herskowitz, 1994a). Not surprisingly, mutants deleted for *fuz7* are defective in the processes dependent on the *a* locus and its pheromones: response of haploids to mating pheromones and maintenance of the filamentous form after mating (Banuett and Herskowitz, 1994a, 1994b). An unanticipated finding is that the *fuz7* gene is also involved in processes that do not require the *a* locus or its pheromones, in particular, induction of tumors in its maize host. One appealing hypothesis is that the putative *fuz7* MEK is a component of a MAPK module necessary for responding to signals from the plant that induce fungal competence for tumor formation (Banuett and Herskowitz, 1994a). These studies offer a first look into the role of signaling pathways in the infectious process of fungal pathogens.

Concluding Comments

The diversity of processes in which MAPKs and their associated activating kinases participate within a unicellular organism is remarkable: mating, developmental responses triggered by starvation, response to increased extracellular osmolarity, maintenance of cell wall integrity, and spore formation. Only in the pheromone response pathways of budding and fission yeasts and in the PKC1 pathway of budding yeast have complete MAPK "modules," with MEKK, MEK, and MAPK, been identified. It will be intriguing to see whether the missing components of this "integral module" will be identified in the HOG (for high osmolarity glycerol response) and sporulation pathways and whether components identified thus far only in the pheromone response pathway, in particular, STE5 and STE20, will be found elsewhere. STE20 homologs are already known in mammalian systems, but there the question is to learn their biological role. Why have some of the components of the MAPK module, for example, a MEKK in the HOG pathway, not yet been found? One possibility is that some of the components in this pathway are functionally redundant, as has been observed for the MAPKs of the pheromone response pathway (FUS3 and KSS1) and for the MEKs of the PKC1 pathway (MKK1 and MKK2). Because of the functional redundancy, simple mutations that inactivate the *KSS1*, *MKK1*, or *MKK2* genes show no phenotype. In fact, these genes were identified by the powerful technique of selecting for high copy number plasmids that overexpress these genes and thereby exhibit an observable phenotype. The flip side of functional redundancy is specificity, and here genetic analysis in yeast has made it possible to demonstrate that many of the components of the different pathways are dedicated to one particular pathway. For example, mutations in the HOG pathway genes *PBS2* or *HOG1* have no effect on any of the other pathways. In contrast, STE7 and STE11 play a role in three different pathways: pheromone response and invasive growth of haploid cells, and pseudohyphal development in **a**/α diploid cells. The use of mutants to assess the function of a MAPK pathway component in different pathways has also been beautifully exploited in nematodes and in Drosophila (see, for example, Biggs et al., 1994).

It has been suggested (Marcus et al., 1994; Choi et al., 1994; Printen and Sprague, 1994) that the STE5 protein may play a role in restricting cross-talk between MAPK pathways. If so, then mutations in the *STE5* gene might relax complementation by analogous components from other organisms and provide a route to identifying and characterizing them. Conservation of the components of the MAPK module between fission and budding yeast has already been exploited to identify a mammalian MEKK (Lange-Carter et al., 1993). Finding a fission yeast homolog to STE5 would greatly facilitate searches for STE5 homologs in other organisms.

We began this review by speaking of MAPK modules, but what does it mean to say module? It was clear from the outset that a module is not an indivisible unit, since mammalian cells have two types of MAPK modules: raf–MEK–MAPK and MEKK–MEK–MAPK. We now see from a comparison of the pheromone response pathway with the invasive growth and pseudohyphal development pathways that two MAPK modules can differ in their MAPKs: all contain STE11 and STE7, but the pheromone response pathway employs FUS3 or KSS1, whereas the other two pathways likely use different MAPKs. The concept of a module comes in part from finding that the MEKK, MEK, and MAPK act directly on each other. It seems possible that the module could be expanded to include, for example, STE20 if several different MEKKs are found to be regulated by STE20 relatives. It is notable that no studies

show association between STE5 and STE20. The discovery of the physical association between STE5 and the components of the pheromone response pathway MEKK–MEK–MAPK, of course, gives concrete physical basis for the idea that these protein kinases function as a module. Given that several components of the pheromone response pathway are present in a complex, there are many questions that remain. What is the stoichiometry of the components in the complex? Are there multiple species of these complexes? What other proteins are present? And, finally, what does STE5 actually do? Is it simply a scaffold?

Studies with yeast provide examples of biological processes in which MEKKs participate: pheromone response and maintenance of cell wall integrity. How these enzymes are activated is not yet clear, although some that may be involved (STE20, STE5, and PKC1) have been identified. It may be appropriate for us to toss the 14–3–3 proteins' hat into this ring; they have been found to play a role in activation of raf, which, after all, is a MEK kinase (see, for example, Freed et al., 1994). Although yeast does not have its own raf, mammalian c-Raf-1 can work in yeast under certain conditions to stimulate a partially active *STE7* MEK (Irie et al., 1994). The ability of c-Raf-1 to work under these circumstances is enhanced by expression of mammalian 14–3–3, and, indeed, a yeast 14–3–3 homolog encoded by the *BMH1* gene can also be demonstrated to be necessary for function of c-Raf-1 (Irie et al., 1994). These observations raise the question of the role of the yeast 14–3–3 gene. Might it be involved in activation of STE11 and other MEKKs? Stay tuned.

None of the studies described here provides a satisfying answer to what is the functional significance of having a kinase cascade. In principle, such a cascade might amplify a signal. The existence of a STE11–STE7–FUS3/KSS1 complex raises the question of whether signal amplification could occur. It will be important to determine whether this complex is stable in vivo or whether its component protein kinases can come and go. As noted above, another rationale for a kinase cascade is that its components are sequestered in some manner to prevent cross-talk with other pathways. Another possibility is that a series of protein kinases provides a mechanism for radiating a signal over different substrate populations: each member of the cascade could have a variety of substrates, not just the protein kinase that it activates. Similarly, the components of a cascade, in principle, could be subjected to multiple inputs. For both the branched input and branched output ideas to be viable, there must be opportunities for proteins other than core components to bind to a protein kinase complex.

Wandering further from the core complex, we see that there may be a diversity of inputs for the MAPK module. Aside from the well-documented serpentine receptors involved in pheromone response, we have seen the possibility of regulation by two-component regulatory systems and glimmerings of regulation by phosphoinositide metabolism and by transcription. The receptors for the HOG and PKC1 systems might be, for example, mechanoreceptors on the cell surface. The nature of the receptors and signals for invasive growth and pseudohyphal development in budding yeast and for development of invading U. maydis ought to be interesting indeed.

The outputs to the MAPK module have so far proven to be somewhat elusive. One substrate for FUS3 and KSS1 is the transcriptional activator STE12, which joins the list of transcription factors activated by MAPKs (see Hill and Treisman, 1995 [this issue of *Cell*]; O'Neill et al., 1994). The suggestion has been made (Costigan et al., 1994) that the yeast HMG1-like proteins NHP6A and NHP6B might be substrates for the *MPK1* MAPK in the PKC1 pathway. The targets for these proteins are unknown. A straightforward route may exist to identify targets for the MAPK in the HOG pathway, since a DNA segment conferring osmoinducibility has been identified. Of course, substrates for MAPKs are not restricted to transcription factors: another substrate for FUS3 is FAR1, which binds to cyclin-dependent kinases to provoke cell cycle arrest. The identification of an inhibitor of initiation factor 4E (PHAS-I) as another substrate for mammalian MAPKs demonstrates the diverse substrates that are to be expected (Lin et al., 1994).

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